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Cystic fibrosis transmembrane conductance regulator (CFTR) regulates embryonic organizer formation during zebrafish early embryogenesis

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ABSTRACT Cystic fibrosis (CF) is associated with the manifestation of a number of medical conditions throughout the body. This prompted us to investigate the etiology of CF from the viewpoint of the embryonic organizer, which is responsible for steering the movement of surrounding cells into specific organs and tissues. In our previous work, we found that a *cftr* mutant had decreased nuclear β-catenin levels in the early embryo at 5 hours post-fertilization (hpf), when the organizer forms. It is known that nuclear β-catenin signaling is essential for the induction of the dorsal organizer. Therefore, we explored the role of *cftr* in the formation of the embryonic organizer in this work. Indeed, the expression of organizer and germ layer markers was significantly affected in *cftr* mutant embryos dependent on Wnt/β-catenin signaling. Furthermore, quantitative proteome analysis revealed that the *cftr* mutant induced significant alteration in the expression of proteins related to many critical biological processes, cellular components, molecular functions, and signaling pathways, except for the Wnt/β-catenin pathway. These findings demonstrate the function of *cftr* in embryonic organizer formation and provide an explanation for why many abnormalities occur in the bodies of CF patients.

KEY WORDS: *CFTR, organizer, early embryogenesis*

Introduction

Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-activated anion channel protein that belongs to ATP-binding cassette (ABC) transporter superfamily (Gadsby *et al.*, 2006). It was first found to be expressed in a wide variety of epithelial tissues (Tizzano *et al.*, 1993). Mutations of *CFTR* cause cystic fibrosis (CF), the most common lethal congenital disease in Caucasians (Quinton, 1999, Riordan, 2008). The hallmark of CF is a defect in electrolyte and fluid transport affecting multiple organ systems with a multitude of clinical manifestations (Quinton, 1999, Riordan, 2008), such as obstructive lung disease (Johannesson *et al.*, 2012, Pezzulo *et al.*, 2012), pancreas exocrine deficiency (Wilschanski and Novak, 2013), CF-related diabetes (Guo *et al.*, 2014), and abnormal gonad function and infertility (Chen *et al.*, 2012, Lu *et al.*, 2012, Xu *et al.*, 2007), which is characterized by progressive organ dysfunction with the development of scarring and fibrosis (Bright-Thomas and Webb, 2002, Labombarda *et al.*, 2016).

Organizers, which comprise groups of cells with the ability to instruct adjacent cells into specific states, represent a key principle in developmental biology. In the context of an embryo, an 'organizer' refers to a group of cells that harbor the ability to instruct fates and morphogenesis in surrounding cells, steering their development into specific organs and tissues. As a result, organizers can position specific tissues and organs relative to each other (Martinez Arias and Steventon, 2018). Therefore, CF patients encountering various additional health issues inspired us to investigate the etiology from the viewpoint of embryonic organizer.

Importantly, in our previous work, we showed that defective *cftr* results in accelerated Dpr1 induced Dvl2 degradation, and thus nuclear β-catenin expression reduction, leading to inactivation

Abbreviations used in this paper: CF, cystic fibrosis; CFTR cystic fibrosis transmembrane conductance regulator.

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of Wnt signaling at the beginning of gastrula period (5 hpf) and impaired hematopoiesis during zebrafish early embryogenesis (Sun *et al*., 2018). β-catenin is the key effector of canonical Wnt signals in the future dorsal blastomeres and it induces the formation of the organizer, which acts as a signaling center for correct embryonic patterning and gastrulation cell movements (Yan *et al*., 2018). So, we ask whether CFTR regulates embryonic organizer formation during early embryogenesis.

In this study, we used a zebrafish *cftr* mutant model established by us to investigate the function of *cftr* in organizer formation during embryo development. Our results showed that the formation of embryonic organizer and germ layers was impaired in *cftr* mutant embryos, suggesting an important role of *cftr* in early embryogenesis and providing an explanation for the multitude of clinical manifestations that occur throughout the body in CF patients.

Results

Dorsal organizer formation and early embryogenesis are impaired in cftr *mutant embryos*

In our previous work, we showed that *cftr* deficiency caused by the mutation or knockdown of this gene results in reduced nuclear β-catenin expression induced by Dvl2 degradation at the beginning of gastrula period (5hpf) (Sun *et al*., 2018). Stage of 50% epiboly (5hpf) is not only the starting time of the zebrafish gastrula period to produce mesoderm and hematopoietic progenitors, but also the key time to induce the formation of embryonic organizer (Thisse and Thisse, 2015).

To determine whether *cftr* plays an important role in organizer development during zebrafish embryogenesis, we continued to perform analysis on our existing *cftr* mutant zebrafish lines *cftr*^{scu102} (http://zfin.org/action/feature/view/ZDB-ALT-190307-1). *cftr*^{scu102} carried a 2-bp deletion in Exon 6, causing a frameshift mutation leading to a premature stop codon at 219 AA (Sun *et al*., 2018).

As revealed by whole-mount *in situ* hybridization (WISH), the dorsal organizer markers *gsc* and *chd* are reduced significantly in *cftr*^{scu102} mutants at the shield stage. Meanwhile, the ventral markers *bmp4* and *eve1* and the epidermis marker *gata2a* also showed decreased expression in mutants at this stage. Furthermore, similarly to the marker genes detected at the shield stage, the mesodermal marker gene *ntla*, the endodermal marker gene *sox17*, and the anterior neuroectoderm marker gene *otx2* were expressed at lower levels at the 70% epiboly stage. Notably, both endoderm (indicated with arrow) and forerunner cell group (indicated with arrowhead) marked by *sox17* showed reduced expression pattern in *cftr*^{scu102} mutant (Fig. 1).

Interestingly, expression of the myogenic marker *myod* was also reduced in mutants at 24 hpf, however, the notochord mesodermal marker *ntla* and the central nervous system marker *sox3* did not show any obvious changes (Fig. 2). These data suggest that in the absence of *cftr*, the development of early embryos is impaired.

Cftr function on embryonic organizer is dependent on Wnt/β-catenin signaling

Given that Cftr deficiency impairs Wnt/β-catenin signaling and hematopoiesis in our previous work(Sun *et al*., 2018), we investigated the functional relationship between Cftr and Wnt/β-catenin on organizer formation. Thus, it appears plausible that the impaired organizer in *cftr* mutant could be due to a deficiency in Wnt/β-catenin. Indeed, injection with *dvl2* or β-*catenin* mRNA ameliorated the impaired organizer caused by deficient Cftr: embryos with normal *gsc* and *chd* expression pattern appeared in *cftr* mutant (Fig. 3). These data suggest that Cftr functions through Wnt/β-catenin on organizer formation.

Proteomics analysis shows aberrant expression of proteins essential for embryo development in cftr mutant embryos at shield stage

Although the reduced nuclear β-catenin levels in *cftr* mutants could provide an explanation for why embryonic organizer formation was impaired, we still need to uncover the underlying molecular mechanism. So, we performed an integrated approach involving TMT labeling and LC-MS/MS to quantify the dynamic changes of the whole proteome of zebrafish embryos at shield stage (6 hpf) (*cftr*^{scu102} mutant vs WT).

In total, 3,381 proteins from embryos were identified in response to *cftr*^{scu102} mutant and WT embryos, among which 2,836 proteins were quantified. All the annotation and quantification

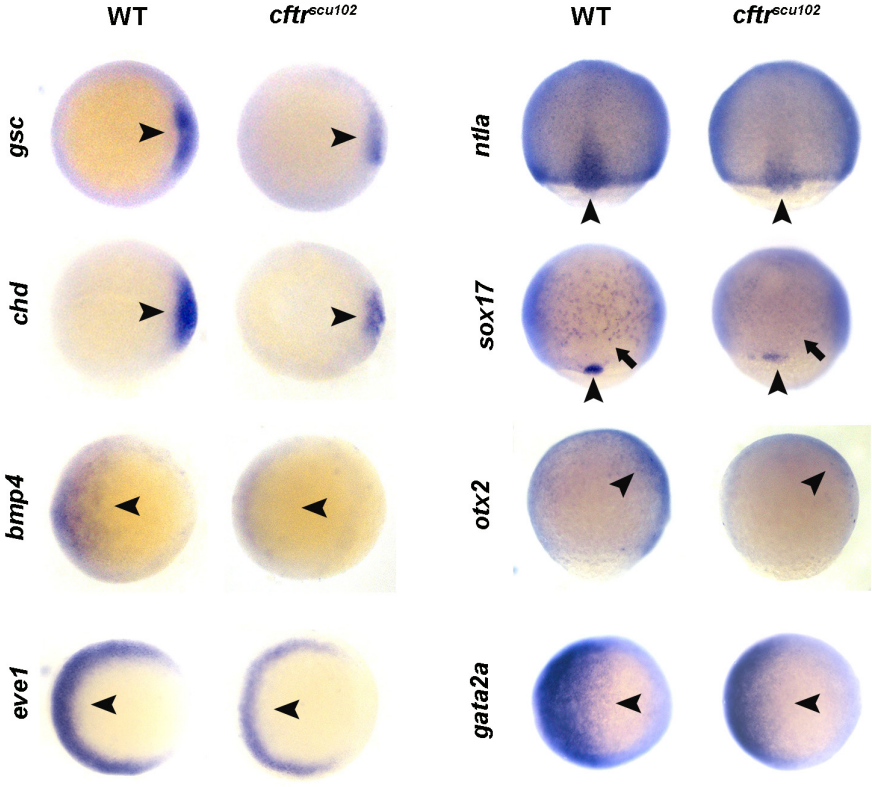

Fig. 1. Embryonic organizer and germ layer marker expression detected by WISH during gastrulation. *Orientation:* ntla *and* sox17*, dorsal views with animal pole to the top;* otx2*, side views with dorsal to the right at 70% epiboly stage; others, top views (*gsc, chd, bmp4, eve1 *and* gata2a*) with dorsal to the right at shield stage. Arrowheads indicate the expression sites of each marker gene.

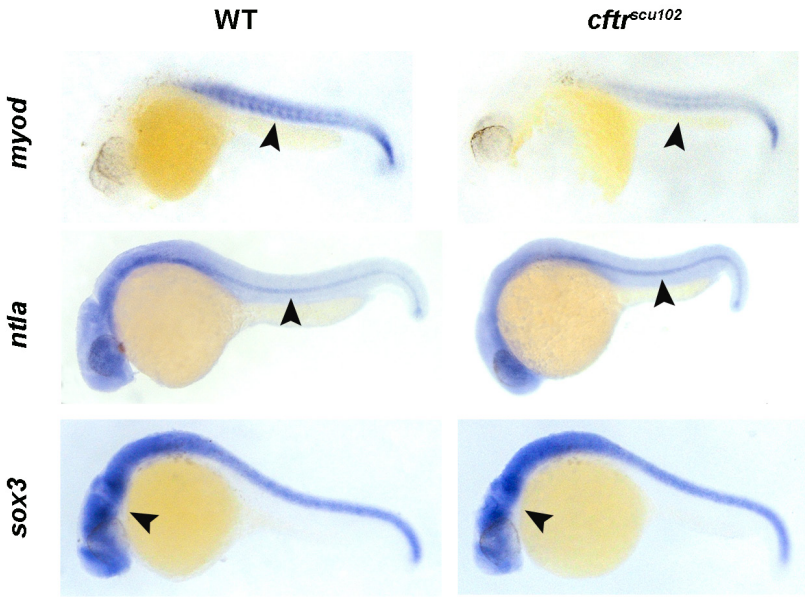

Fig. 2. Marker expression of *myod, ntla* and *sox3* at 24 hpf. *Lateral views with anterior to the left. Arrowheads indicate the expression sites of each marker gene.*

information were presented in the Supplemental Table S1. Relative quantitation of proteins was divided into two categories. Quantitative ratio over 1.2 was considered up-regulation while quantitative ratio less than 1/1.2 was considered as down-regulation. Results showed that *cftr*^{scu102} mutant induced 190 differentially expressed proteins (117 up-regulated and 73 down-regulated) (Supplemental Table S2).

To characterize the function of these altered proteins, a Gene Ontology (GO)-based classification analysis of the ontology of biological processes, cellular components and molecular functions was performed, and it revealed widely different distributions between *cftr*^{scu102} mutant and WT embryos (Fig. 4A and Supplemental Table S3). With regard to biological processes, we discovered that the proteins in response to *cftr*^{scu102} mutant showed enrichment of cellular processes, single-organism processes, metabolic processes, biological regulation, developmental processes, multicellular organismal process, response to stimulus, cellular component organization or biogenesis, localization, etc. Molecular function-based enrichment results revealed that binding, catalytic activity, molecular function regulators, structural molecule activity, transporter activity, etc. in regulated proteins were enriched in *cftr*^{scu102} mutant embryos. In the cellular component category, cytoplasm, extracellular, nucleus, mitochondria, plasma membrane, endoplasmic reticulum, cytoskeleton, etc. were enriched in the regulated proteins (Fig. 4B and Supplemental Table S4).

To further characterize the mechanism of these altered proteins, KEGG pathway-based classification analysis on the signaling pathways was performed, and it revealed a widely different distribution in *cftr*^{scu102} mutant embryos compared to WT, including metabolic pathways, biosynthesis of amino acids, glycolysis/gluconeogenesis, amino sugar and nucleotide sugar metabolism, carbon metabolism, ECM-receptor interaction, pentose phosphate pathway, pentose and glucuronate interconversions, glutathione metabolism, fructose and mannose metabolism, AGE-RAGE signaling pathway in diabetic complications, fatty acid metabolism and metabolism of xenobiotics by cytochrome (Fig. 4C and Supplemental Table S5).

In conclusion, quantitative analysis of the global proteome between *cftr*^{scu102} mutant and WT embryos indicated that *cftr* mutation significantly impacts embryos, resulting in a remarkable alteration of many critical biological processes, cellular components, molecular functions and signaling pathways, except for the Wnt/β-catenin pathway.

Discussion

WISH detected both maternal and zygotic *cftr* expression throughout early development. This expression patterns suggest that *cftr* plays a role in early axis formation(Sun *et al.*, 2018). Loss of *cftr* function in zebrafish model leads to destruction of the embryonic hematopoiesis (Sun *et al.*, 2018), the migration of primordial germ cells (Liao *et al.*, 2018), cardiac development (our submitted data), the organ laterality defects, the lumen expansion, function of Kupffer's vesicle (Navis *et al.*, 2013), exocrine pancreas (Navis and Bagnat, 2015) and the gut tube (Bagnat *et al.*, 2010). These cystic fibrosis phenotypes mirror the symptoms in the human disease.

While raising *cftr* mutants to adulthood, a large percentage of the mutants are lost beginning around 10 dpf (Liao *et al.*, 2018). Furthermore, the *cftr* mutants begin to experience growth restriction coincident with the decreased survival (Navis and Bagnat, 2015), suggesting the impaired body development at early embryogenesis.

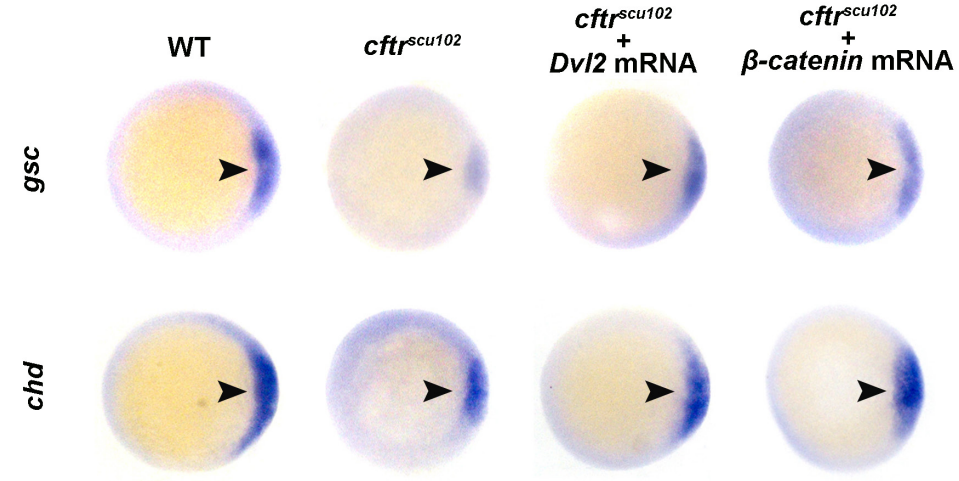

Fig. 3. Effects of *dvl2* or *β-catenin* mRNA in rescuing organizer defects in *cftr* mutant. *Embryos shown are top views with dorsal to the right at shield stage.*

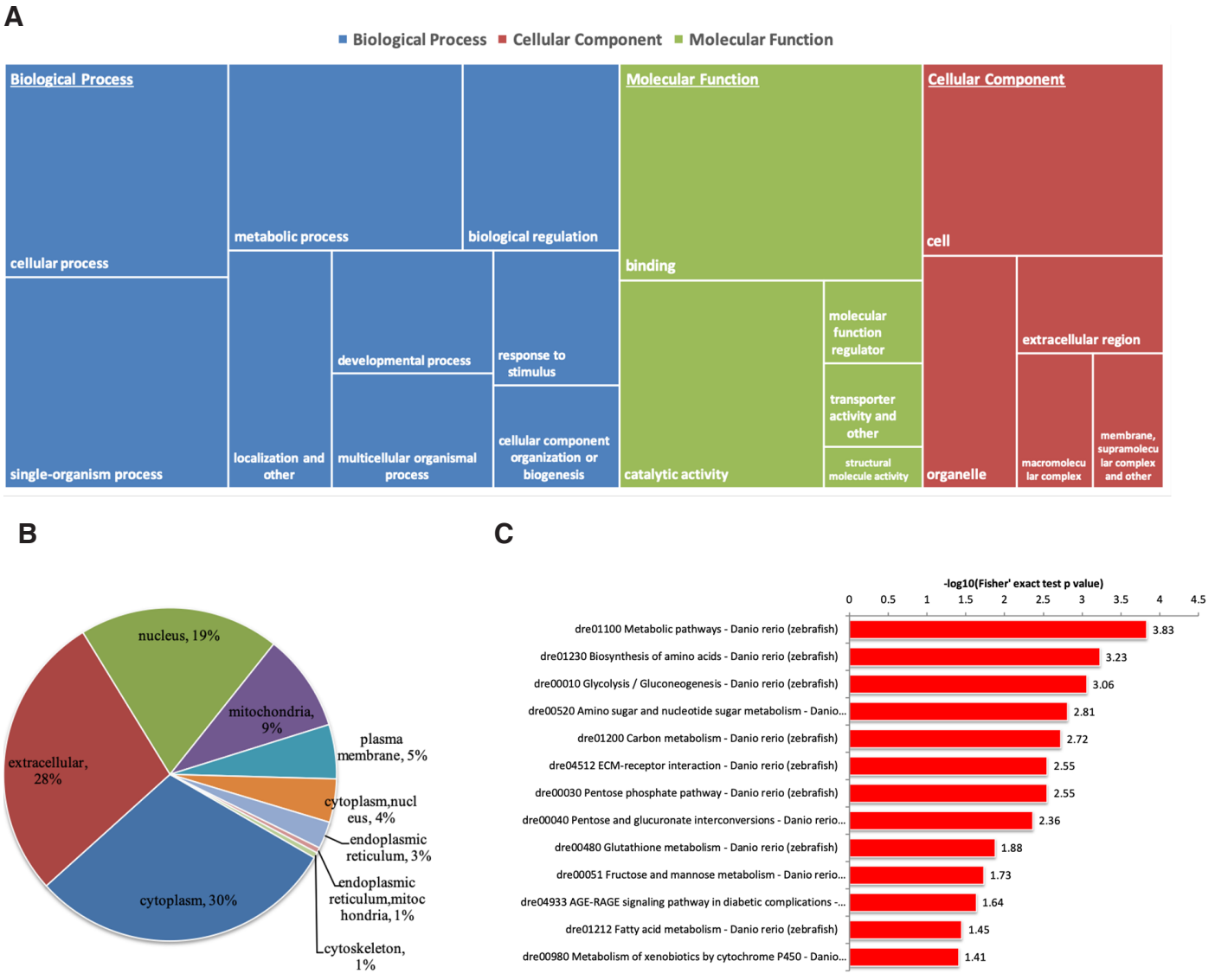

Fig. 4. The cftr mutant induces significant alterations in the proteome of embryos at the shield stage (*cftr*^{scu102} mutant vs WT). (A) *Gene Ontology (GO)-based enrichment analysis of regulated proteins on the ontology of biological processes, cellular components, and molecular functions.* **(B)** *Cellular component category analysis of regulated proteins.* **(C)** *KEGG pathway classification analysis of proteomic data.*

Navis *et al.,* described that proper midline expression of *ntl* and *lefty1* indicates that midline integrity is not perturbed in their *cftr* mutants. In addition, the notochord and floorplate of their *cftr* mutants appeared to be completely intact at 24 hours post-fertilization (hpf) as judged by DIC microscopy (Navis *et al.*, 2013). Consistently, we also observed that the midline, notochord and floorplate marked by *ntla* and *sox3* were not impaired in embryos at 24 hpf.

Unfortunately, the identified quantitative proteins were fewer than expected in the embryonic proteomics analysis, because of high proportion of yolk proteins in early embryos. Even so, we identified 3,381 proteins and quantified 2,836 proteins and found 190 differentially expressed proteins. GO function classification analysis revealed that a wide range of proteins is regulated by *cftr*^{scu102} mutants, affecting many critical biological processes, cellular components, molecular functions, and signaling pathways except for the Wnt/β-catenin pathway.

Materials and Methods

Ethical approval and ethics statement

All experiments in this study were in accordance with the "Guide for the Care and Use of Laboratory Animals" (Eighth Edition, 2011. ILARCLS, National Research Council, Washington, D.C.) and were approved by the Animal Care and Use Committee of West China Second University Hospital, Sichuan University (Approval ID: HXDEYY20131021).

Zebrafish lines and embryos

Wildtype (WT) AB strain, *cftr*^{scu102} (http://zfin.org/action/feature/view/ZDB-ALT-190307-1) fish lines were utilized. Staging of the embryos was carried out as previously described (Sun *et al.*, 2018).

Proteomics analysis of embryos

Quantitative proteome analysis was performed by PTM-Biolabs (HangZhou) Co., Ltd., detailed materials and methods was same to our

previous work(Liu *et al*., 2017).

Assays and statistics

Zebrafish embryo whole-mount *in situ* hybridization, grayscale measurement, and statistics were performed as previously described(Sun *et al*., 2018).

Declaration of interest

All the authors listed declare no competing financial interests and have approved the manuscript that is enclosed.

Availability of data and materials statements

All data generated or analyzed during this study are included in this published article.

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Author Contributions statement

H.S. and Y.L. conceived and designed the experiments; H.S. and Z.L. performed the experiments; H.S. and Y.L. analyzed the data; H.S. wrote the paper.

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